# NOVEL PHOSPHATE-BINDING PROTEIN, PHARMACEUTICAL COMPOSITIONS CONTAINING IT AND USES THEREOF

A subject of the present invention is a novel phosphate-binding protein, obtained from human serum, pharmaceutical compositions containing it as well as uses thereof, in particular within the framework of the treatment of hyperphosphataemia and cardiovascular diseases or arthritis.

Phosphate is a very important molecule involved in numerous biological mechanisms. Phosphate is found in particular in the phospholipids, in the energy-production mechanism (ATP, ADP), in the cell signalling processes and in the composition of the genetic material in the bones (in the form of calcium phosphate).

Hyperphosphataemia is a pathology linked to an excess of phosphate in the organism and causes in particular an increase in the risks of cardiovascular diseases, by promoting the processes of atherosclerosis and calcification of the arteries (Dorozhkin and Epple, 2002; Amann et al., 2003; Blazheevich et al., 1975). As calcification takes place in the joints, hyperphosphataemia can also cause arthritis (pseudogout).

The calcium phosphate salts produced in the serum during hyperphosphataemia precipitate in the soft tissues with ectopic calcification in different tissues: vessels (cerebral or cardio vascular accidents), joints (pseudogout), lens, renal interstitium (nephrocalcinosis), sub-cutaneous (pruritis), pulmonary, and pancreatic.

Thus, half the deaths of individuals suffering from renal insufficiency is due to cardiovascular diseases linked to hyperphosphataemia. In this regard, certain phosphate chelating agents which complex the phosphate in the intestinal lumen are currently used as medicaments. However, not all these chelating agents are physiological. This results in certain complications or restrictions as to their use.

Preparations containing magnesium are limited by the appearance of digestive disorders (diarrhoea) and are to be proscribed because of the risk of hypermagnesaemia. Similarly, the prescription of aluminium hydroxide, long used because of its effectiveness, must be avoided, or at least limited to very short periods, because of the risk of aluminium intoxication (microcytic hypochromic anaemia, osteomalacia, myopathy, dementia).

The prescription of calcium salts is the best means for correcting both hypocalcaemia and hyperphosphoraemia, making it possible on the one hand to increase

the quantity of calcium absorbed by the small intestine in spite of the calcitriol deficiency, and on the other hand to complex the phosphorus in the intestinal lumen in the form of calcium phosphate which is eliminated in the faeces. However, the major drawback of the chelating agents containing calcium is that of inducing hypercalcaemia, which, in certain series, has been noted in 20% of patients. This risk has lead to the development of other products capable of limiting hyperphosphoraemia.

The medicament most used at present is Renagel® (Ramsdell; 1999). This is a non-absorbable cationic polymer, capable of chelating phosphate.

The purpose of the present invention is to provide a novel physiological protein chelating agent binding to phosphate, not requiring the use of other ions which can lead to complications and offering wider use perspectives than current chelating agents.

The present invention relates to a protein characterized in that it comprises or is constituted by:

– the sequence SEQ ID NO: 1,

– or any sequence derived from the sequence SEQ ID NO: 1, in particular by substitution, suppression or addition of one or more amino acids, providing that said derived sequence binds to phosphate,

– or any sequence homologous to the sequence SEQ ID NO: 1, preferably having a homology of at least approximately 80% with the sequence SEQ ID NO: 1, providing that said homologous sequence binds to phosphate,

– or any fragment of one of the sequences defined above, providing that said fragment binds to phosphate, in particular any fragment being constituted by at least approximately 20 contiguous amino acids in the sequence SEQ ID NO: 1.

The present invention relates to a protein as defined above, characterized in that it comprises or is constituted by:

– the sequence SEQ ID NO: 2 or the sequence SEQ ID NO: 3,

– or any sequence derived from the sequence SEQ ID NO: 2 or SEQ ID NO: 3, in particular by substitution, suppression or addition of one or more amino acids, providing that said derived sequence binds to phosphate,

– or any sequence homologous to the sequence SEQ ID NO: 2 or SEQ ID NO: 3, preferably having a homology of at least approximately 80% with the sequence SEQ ID NO: 2 or SEQ ID NO: 3, providing that said homologous sequence binds to phosphate,

– or any fragment of one of the sequences defined above, providing that said fragment binds to phosphate, in particular any fragment being constituted by at least

approximately 20 contiguous amino acids in the sequence SEQ ID NO: 2 or SEQ ID NO: 3.

The sequence SEQ ID NO: 2 corresponds to the human phosphate-binding protein. This novel protein has been isolated in human plasma and its three-dimensional structure shows that it belongs to the "phosphate binding protein" (PBP) class. It is also called hereafter HPBP (human phosphate binding protein).

The sequence SEQ ID NO: 3 corresponds to a protein homologous to the protein sequence SEQ ID NO: 2, having a percentage of identity of approximately 90% with the sequence SEQ ID NO: 2, and having the same phosphate-binding properties as the sequence SEQ ID NO: 2.

The phosphate-binding property of the sequences of the invention can be verified by the following phosphate-binding test by radioactive labelling:

The protein is bound to a nitrocellulose membrane (dot blot by aspiration). The membrane is left to incubate in a radioactive buffer ($^{32}P$ (10 mCi/ml, Amersham-Biosciences) 2M; Tris 50 mM; pH 8.0)

The membrane is rapidly rinsed 2 × 1 min in a Tris 50 mM buffer, pH 8.0. By exposing a photographic film with the membrane (approximately 45 min) it is possible to detect the zones which bind the radioactive phosphate (see Figure 3 hereafter).

The present invention also relates to a nucleotide sequence encoding a protein as defined above.

The present invention also relates to a recombinant vector, in particular plasmid, cosmid, phage or virus DNA, containing a nucleotide sequence as defined above.

According to an advantageous embodiment, the present invention relates to a recombinant vector as defined above, containing the elements necessary for the expression in a host cell of the polypeptides encoded by the nucleotide sequence as defined above, inserted into said vector.

The present invention also relates to a host cell, chosen in particular from bacteria, viruses, yeasts, fungi, plants or mammal cells, said host cell being transformed, in particular using a recombinant vector as defined above.

The present invention also relates to a pharmaceutical composition comprising as active ingredient a protein as defined above, in particular SEQ ID NO: 2 or SEQ ID NO: 3, in combination with a pharmaceutically acceptable vehicle.

The present invention also relates to a pharmaceutical composition as defined above, in which the protein of the invention, in particular SEQ ID NO: 2 or SEQ ID

NO: 3, is in combination with a variant of the paraoxonase protein, having a paraoxon hydrolysis activity.

Among the variants of paraoxonase, there can be mentioned the variants PON1, PON2, PON3, of human or non-human origin, such as SEQ ID NO: 4 (human PON1; Hassett et al., 1991), SEQ ID NO: 5 (human PON2; Primo-Parmo et al., 1996), SEQ ID NO: 6 (human PON3; Reddy et al., 2001), SEQ ID NO: 7 (rabbit PON1; Hassett et al., 1991), SEQ ID NO: 8 (rat PON1; Rodrigo et al., 1997), SEQ ID NO: 9 (mouse PON1; Sorenson et al., 1995), SEQ ID NO: 10 (mouse PON2; Primo-Parmo et al., 1996) and SEQ ID NO: 11 (mouse PON3; Primo-Parmo et al., 1996).

The present invention also relates to the use of a protein as defined above, in particular SEQ ID NO: 2 or SEQ ID NO: 3, for the preparation of a medicament intended for the prevention or treatment of diseases linked to hyperphosphataemia, such as cardiovascular diseases and arthritis (pseudogout).

The term "hyperphosphataemia" designates an excess of phosphate in the organism. More precisely, hyperphosphataemia is defined by an increase in the phosphate concentration in the plasma above 1.44 mmol/l (45 mg/l), said quantity being obtained by assay of the total phosphate (assay by colorimetric method is carried out after a mineralization process).

According to an advantageous embodiment, the protein of the invention can be administered in intravenous form in order to be able to bind a maximum quantity of phosphate over a long period, of the order of a week. By subsequently eliminating the protein, a large quantity of phosphate is thus rapidly eliminated. This makes it possible to space out and reduce the periods of dialysis.

The present invention relates more particularly to the use of a protein as defined above, in particular SEQ ID NO: 2 or SEQ ID NO: 3, within the framework of the prevention or treatment of cardiovascular diseases.

The present invention also relates to the use of a protein according to the invention, in particular of the protein represented by the sequence SEQ ID NO: 2 or SEQ ID NO: 3, in combination with a protein such as a variant of the paraoxonase protein, within the framework of the prophylaxis or treatment of intoxications caused by insecticides or nerve agents, such as soman, VX, tabun or sarin, or within the framework of the treatment of atherosclerosis.

The present invention also relates to a combination product comprising at least one protein as defined above, in particular SEQ ID NO: 2 or SEQ ID NO: 3, and at least

one variant of the paraoxonase protein, for simultaneous or separate use or use spread over time intended for the prophylaxis or treatment of intoxications caused by insecticides or nerve agents, such as soman, VX, tabun or sarin.

The combined use of the protein of the invention, in particular SEQ ID NO: 2, with a variant of the paraoxonase protein, makes it possible to increase the stability of the paraoxonase, in particular within the framework of the prophylaxis or treatment of the intoxications caused by insecticides or nerve agents.

The present invention also relates to a protein assay method as defined above, characterized in that it comprises the following stages:

– rabbit monoclonal antibodies directed against different epitopes of the protein of the invention (anti-HPB) are fixed on a plate and the human serum to be analyzed containing said protein (HPB) is applied to the above-mentioned plate,

– the plate is rinsed and washed,

– antibodies directed against rabbit antibodies (anti-IGrabbit-per) marked with peroxidase are applied to the plate over 30 minutes, in order to form a ternary complex between a rabbit monoclonal antibody, the protein according to the invention and an above-mentioned antibody directed against a rabbit antibody (anti-HPB – HPB – anti-IGrabbit-per),

– the plate is rinsed and washed,

– the peroxidase fixed to the plate is reacted with its substrate (commercially available kit, Chemiluminescent Peroxidase Substrate (Sigma)) and the reaction is stopped at the end of 30 minutes with 3,3',5,5'-tetramethylbenzidine (TMB, Sigma),

– the optical density of the product formed in the preceding stage is measured at 450 nm using a spectrophotometer, and comparison of this measurement with a standard curve makes it possible to determine the concentration of the protein according to the invention (HPB) present in the serum.

Thus, the above-mentioned assay method uses an ELISA-type immunoassay method (Engvall et al., 1971).

Other methods can be used to assay the concentration of the protein of the invention in the plasma such as:

– electrophoretic methods, or

– the quantification of its activity.

The present invention also relates to the application of the assay method as defined above

to the *in vitro* diagnosis of diseases linked to hyperphosphataemia in particular when the quantity of protein as defined above, in particular SEQ ID NO: 2 or SEQ ID NO: 3, assayed according to the method as defined above, is less than the quantity of this protein normally present in the blood of a healthy individual, or

to the *in vitro* diagnosis of diseases linked to hypophosphataemia in particular when the quantity of protein as defined above, in particular SEQ ID NO: 2 or SEQ ID NO: 3, assayed according to the method as defined above, is greater than the quantity of this protein normally present in the blood of a healthy individual, or

to the *in vitro* diagnosis of an individual's predisposition to such pathologies.

The level of the protein according to the invention is an indicator of predisposition to a risk of cardiovascular disease. Thus, individuals having a low level of said protein will have a higher level of free phosphate which will precipitate with the calcium in the plasma to form calcium phosphate plates, which is a factor aggravating in particular the risks of cardiovascular diseases or arthritis.

An abnormal level of this protein is also the sign of an existing pathology. For example hyperphosphataemia can trigger an increased production of protein in order to limit the phosphate level. A low level can also reveal a dysfunction.

The present invention also relates to the application as defined above to the *in vitro* diagnosis of diseases linked to hyperphosphataemia such as cardiovascular diseases, in particular cardiovascular diseases linked to the formation of atheroma plaques, or to the *in vitro* diagnosis of an individual's predisposition to develop one of the above-mentioned diseases.

The present invention also relates to the application as defined above to the *in vitro* diagnosis of diseases linked to hypophosphataemia, or to the *in vitro* diagnosis of an individual's predisposition to develop these diseases.

Among the clinical or physiological signs characterizing diseases linked to hypophosphataemia, there can be mentioned:

- a demineralization of the bones,
- the muscular manifestations of hypophosphataemia which comprise a proximal myopathy affecting the skeletal muscle and dysphagia and an ileus affecting the smooth muscles,
- cardiopulmonary deficiencies due to the lack of ATP, and
- metabolic encephalopathy.

## LEGENDS TO THE FIGURES

Figure 1 represents an SDS-PAGE gel of the final fractions within the framework of the purification of human paraoxonase and the protein of the invention SEQ ID NO: 2.

Column A corresponds to the molecular weight marker and columns B, C and D to three different purifications originating from different bags of human plasma. They all three contain human paraoxonase and the phosphate-binding protein.

Figure 2 represents the diagrammatic structure of the protein of the invention SEQ ID NO: 2 to which a phosphate molecule is bound.

Figure 3 corresponds to a test of phosphate binding by the protein of the invention SEQ ID NO: 2.

Columns A to F correspond to different batches of purification of the protein of the invention originating from different bags of human plasma; column G to lysozyme 1 mg/ml and column H to β-lactoglobulin.

Figure 4 represents a two-dimensional electrophoresis gel of a mixture of the protein of the invention SEQ ID NO: 2 and paraoxonase.

Figure 5 represents the molecular coordinates of the crystallized protein of the invention SEQ ID NO: 2.

## EXPERIMENTAL PART

Isolation of the protein

The protein SEQ ID NO: 2 is obtained from human plasma according to the following method of Gan et al. (1991):

The protein SEQ ID NO: 2 is purified from bags of frozen plasma (~200 ml) supplied by the *Etablissement de Transfusion Sanguine* of Lyon-Beynost. The fibrin clot, formed by the addition of 1 M (1% v/v) of $CaCl_2$ to the plasma is separated from the serum by filtration. The serum is then mixed with 400 ml of affinity gel (Cibacron 3GA-Agarose, C-1535, Sigma) equilibrated with a buffer A (Tris/HCl 50 mM, $CaCl_2$ 1mM, NaCl 4M, pH 8). Under these conditions, mainly the HDLs ("high density lipoproteins") are adsorbed. After incubation for 6 to 8 hours, the proteins not adsorbed on the gel are eliminated by filtration on a fritted disc of porosity No. 2. This washing is carried out until no more protein is detected in the eluate (UV absorption at 280 nm). The gel is then equilibrated with a buffer B (Tris/HCl 50 mM, $CaCl_2$ 1mM, pH 8) then placed in an XK 50/30 column (Pharmacia). The elution is carried out by adding 1g/l of sodium deoxycholate and 0.1% of triton X-100 to buffer B. The fractions showing an arylesterase activity are injected onto 50 ml of an anion-exchange gel (DEAE Sepharose Fast Flow, Pharmacia) arranged in an XK 26/70 column (Pharmacia) and equilibrated with buffer B and 0.05% triton X-100. The elution is carried out by NaCl gradient. A first plateau is reached at 87.5 mM of NaCl in order to eliminate the apo A-I, a protein linked to paraoxonase, and the majority of the contaminating proteins. Human paraoxonase (PON1) is approximately eluted at a concentration of 140 mM of NaCl. All the fractions retained show a paraoxonase and arylesterase activity, these activities being verified according to the tests mentioned below. The eluted fractions are not brought back together. The SDS-PAGE gels of the fractions obtained show bands comprised between 38 kDa and 45 kDa (see Figure 1). Each purification does not always result in the same apparent mass distribution. This slight heterogeneity can be explained by the presence of 2 glycosylated chains on the PON1.

In addition to the PON1 in these batches another protein has been isolated by crystallization, by substituting C12-maltoside for triton and using ammonium sulphate as precipitating agent. The crystals obtained are those of an unknown protein characterized by radiocrystallography and corresponding to the sequence SEQ ID NO: 2

of the invention. Crystallization is at present the only existing method for purifying this protein.

The paraoxonase activity is measured in a glycine 50 mM/NaOH, $CaCl_2$ 1 mM buffer, in the presence of 1 M NaCl, pH 10.5 and is determined by means of a double beam spectrophotometer (Shimadzu UV 160A) thermostatically controlled at 25°C. The speed of hydrolysis is determined according to the variation of absorbance at 412 nm, corresponding to the formation of p-nitrophenol released by the hydrolysis of paraoxon, as a function of time, $\varepsilon = 18290\ M^{-1}cm^{-1}$ (Smolen, 1991).

The arylesterase activity is measured in a tris 50 mM/HCl, $CaCl_2$ 1mM buffer, pH 8 and is determined by means of a double beam spectrophotometer (Shimadzu UV 160A) thermostatically controlled at 25°C. The speed of hydrolysis is determined according to the variation in absorbance at 270 nm, corresponding to the formation of phenol released by the hydrolysis of phenyl acetate, as a function of time, $\varepsilon = 1310\ M^{-1}cm^{-1}$ (Smolen, 1991).

Structure

The structure of the crystallized protein SEQ ID NO: 2 was obtained by X-ray crystallography. The structure at 1.9 Å resolution was obtained by the SIRAS (Single Isomorphous Replacement and Anomalous Scattering) method (Figure 2).

The X-ray diffraction data were collected on the BM30 line of the ESRF (Grenoble).

A heavy atom salt derivative was obtained by soaking a crystal in a solution containing uranium salts.

The images were integrated, scaled and combined with the XDS2000 programs (Kabsch, 1993) and the CCP4 suite (COLLABORATIVE COMPUTATIONAL PROJECT, NUMBER 4. 1994. "The CCP4 Suite: Programs for Protein Crystallography". Acta Cryst. D50, 760-763).

The CNS (BRUNGER, 1998) and SnB (Weeks, 1999) programs were used in order to locate the uranium atoms. The SHARP program (Copyright © 2001-2002 the Buster Development Group) was used in order to obtain the phases by the SIRAS technique.

372 amino acids were constructed automatically in the electronic density map by the ARP/wARP (Perrakis, 1997) program. This first model was then refined by the CNS program.

Because of the very good quality of the electronic density map, it was possible to assign 80% reliability to the primary sequence of the protein. It was also possible to locate a phosphate molecule.

The structure obtained does not at all correspond to human paraoxonase. The sequencing obtained by identifying the amino acids from the electronic density indicates that neither this human protein nor its gene have been described previously. It is therefore a novel protein.

The structure of the protein of the invention exhibits a very strong homology with the phosphate-binding protein of *Escherichia coli*. This protein in this bacterium serves to transport the phosphate across the periplasm. It is found in many prokaryotes but in no eukaryote.

The electronic density also showed that a phosphate molecule was bound to the novel protein of the invention, in the same manner as in that of *Escherichia coli*.

Thus, it can be concluded that the protein of the invention characterized from human plasma has a very strong homology with the bacterial protein and that it is capable of binding phosphate and transporting it.

Sequencing

***Digestion in the gel***

The paraoxonase-HPBP mixture was separated by electrophoretic gel with SDS-PAGE (without heating). Several bands corresponding to HPBP in the region of 70 kDa were cut out.

The digestion of the protein contained in these bands was carried out by means of the automatic digestion system, MassPrep Station (Waters Manchester, UK). The gel bands were washed twice with 50 µl of a solution of 25 mM of ammonium bicarbonate ($NH_4HCO_3$) and 50 µl of acetonitrile. The cysteines were reduced with 50 µl of a 10 mM dithiothreitol solution at 57°C and acylated with 50 µl of 55 mM iodoacetamide. After dehydration with acetonitrile, the protein was digested enzymatically with 10 µl of modified porcine trypsin at 12.5 ng/µl (Promega, Madisson, WI, U.S.A) or with lys-C of Lysobacter enzymogenes (Roche Applied Science, Penzberg, Germany) in 25 mM of $NH_4HCO_3$. The digestion is carried out overnight at ambient temperature. The cleaved peptides were extracted with a 60% acetonitrile solution and 5% formic acid.

***Mass spectrometry analysis***

*MALDI-MS and MALDI-MS/MS*

MALDI-TOF mass measurements were carried out on an Ultraflex™ TOF/TOF (Bruker, Daltonik GmbH, Bremen, Germany). This instrument was used with a maximum acceleration voltage of 25 KV in reflectron mode. The sample was prepared with the standard drop preparation dried over the stainless steel target using α-cyano-4-hydroxycinnamic acid as matrix.

The external calibration of the MALDI-MS spectrum was carried out using only the peaks of the monoisotopic charges of a known solution of peptides (bradykinin 1-7 (m/z = 757.400), human angiotensin II (m/z = 1046.542), human angiotensin I (m/z = 1296.685), substance P (m/z = 1347.735), bombesin (m/z = 1619.822), renin (m/z = 1758.933), ACTH 1-17 (m/z = 2093.087) and ACTH 18-39 (m/z = 2465.199)). The masses of the monoisotopic peptides were automatically annotated using the Flexanalysis 2.0 program.

The MS/MS spectra were obtained by analysis of the metastable ions obtained by "Laser-Induced Decomposition" (LID) of a sectioned ion precursor, without additional collision in the gas phase. The ion precursor was accelerated to 8 kV and was selected by means of a timed ion gate. The fragments were further accelerated by 19 kV in the LIFT cell and their masses measured after passing the ion reflector.

The *de novo* sequencing of each of these MS/MS spectra was carried out with the Full DeNovo Sequencing program (Biotools, Bruker Daltonik GmbH, Bremen, Germany).

*Nano LC-MS/MS*

Nano LC-MS/MS analysis was carried out using a CapLC (Waters, Manchester, UK) coupled to a time-of-flight mass spectrometer accelerated by an Q-TOF II orthogonal hybrid quadrupole (Micromass, Manchester, UK). Separation by reversed-phase chromatography was carried out with capillaries (Pepmap C18, 75 µm i.d., 15 cm long, LC Packings) under a flow of 200 nL/min, kept constant by means of a partition pre-column. The calibration was carried out using 2 pmol/µl of GFP.

The mass data acquisition was controlled by the MassLynx program (Micromass, Manchester, UK) which automatically switches between the MS mode and the MS/MS mode.

The MS/MS spectra generated were individually sequenced *de novo* in order to obtain the partial or complete sequence. These interpretations were made using the PepSeq program (MassLynx, Micromass) and the PEAKS Studio program (Bioinformatics Solutions, Waterloo, Canada) which are capable of completely processing a .pkl file with a *de novo* automatic sequencing on each MS/MS spectrum.

Phosphate binding

Phosphate binding by the protein of the invention SEQ ID NO: 2 was demonstrated according to the following test:

200 µl of the protein of the invention SEQ ID NO: 2 (columns A-F of Figure 3), or 1 mg/ml lysozyme (column G) or of β-lactoglobulin was applied to nitrocellulose (dot blot by aspiration).

The mixture is incubated for 2 hours 30 minutes in a mixture comprising: tris 50 mM; pH 8.0; $^{32}P$ (10 mCi/ml) 2 mM.

Rinsing was then carried out twice for 1 minute with tris 50 mM at pH 8.0, then the mixture is exposed at ambient temperature for 45 minutes.

It is then noted (see Figure 3) that the protein of the invention has bound the radioactive phosphate (columns A to F), whereas the test controls have not bound it (columns G and H).

Role and use of the protein SEQ ID NO: 2

For assaying the concentration of this protein in the plasma the methods which can be used are:

- the electrophoretic methods,
- the purification of the protein,
- the quantification of its activity,
- the immunoassay of the protein using polyclonal/monoclonal antibodies directed against the protein.

Combination with paraoxonase

*Two-dimensional electrophoresis*

The purified proteins (40 µg) as described previously in the protocol are mixed with 100 µL of a solution containing 9.8 M of urea, 4% (v/v) triton X100, 2 mM tributyl

phosphine, 0.2 % (v/v) ampholine 3-10 (Bio-Lytes 3 -10; Bio-Rad), and 0.001% (m/v) bromophenol blue. Ready-to-use polyacrylamide gel strips (IPG-Strips; Bio-Rad) (T: 4 %; C: 3 %) are used. Ampholines were bound to the polyacrylamide in a covalent manner so as to have a pre-established linear pH gradient. The pH gradient used is between 3.0 and 10.0.

*1. Isoelectric focusing (IEF)*

The strips are placed in contact with the protein samples in the Protean IEF Cell device (Bio-Rad) and actively rehydrated (50 V constant) for 15 hours at 20°C. Isoelectric focusing is then carried out in 3 stages at 20°C. Firstly, a low voltage of 250 V is applied for 15 minutes; secondly, a rise in gradient from 250 to 4000 V (amperage limited by 50 μA strip) is programmed over 2 hours. Thirdly, the voltage is held constant at 4000 V for 4 hours. After migration, the strips are stored at -20 °C.

According to the preceding purification protocol, the HPBP protein of the invention is co-purified with human paraoxonase (PON) (Fokine et al., 2003). By making a two-dimensional gel with the above protocol, 2 spots were identified by N-terminal sequencing as being respectively the protein of the invention HPBP and human paraoxonase (see Figure 4). The two proteins have approximately the same molecular mass (approximately 40 kDa) and distinct isoelectric points, 6.9-8.5 for HPBP and 4-5 for PON1. Taking account of the fact that it has been necessary to use drastic conditions in order to succeed in separating the 2 proteins (9M of urea and 4% triton) on gel and that the 2 proteins which have very different isoelectric points remain co-purified after passage through an anion exchange column (DEAE sepharose), it is concluded that they are combined by forming a complex.

## BIBLIOGRAPHICAL REFERENCES


– Amann K., Tornig J., Kugel B., Gross M.L., Tyralla K., El-Shakmak A., Szabo A., Ritz E. (2003) *Kidney Int.* **63**(4): 1296-1301;

– Blazheevich N.V., Spirichev V.B., Pozdniakov A.L. (1975) *Kardiologiia.* **15**(6): 67-71;

– Brunger A.T., Adams P.D., Clore G.M., Delano W.L., Gros P., Grosse-Kunstleve R.W., Jiang J.-S., Kuszewski J., Nilges N., Pannu N.S., Read R.J., Rice L.M., Simonson T. et Warren G.L. (1998) *Acta Cryst.* **D54**: 905-921;

– Dorozhkin S.V., Epple M. (2002) *Angew Chem Int Ed Engl.* **41**(17): 3130-46;

– Engvall E., Jonsson K., Perlmann P. (1971) *Biochim Biophys Acta.* (1971) **251**: 427-34;

– Fokine A., Morales R., Contreras-Martel C., Carpentier P., Renault F., Rochu D., Chabriere E. (2003) *Acta Crystallogr D.* **59,** 2083-7;

– Gan, K.N., Smolen, A., Eckerson, H.W. & La Du, B.N. (1991). *Drug Metab Dispos.* **19**, 100-106;

– Hassett, C., Richter, R.J., Humbert, R., Chapline, C., Crabb, J.W., Omiecinski, C.J. et Furlong, C.E. (1991) *Biochemistry* **30**(42), 10141-10149;

– Kabsch W. (1993) *J. Appl. Cryst.* **26**: 795-800;

– Perrakis, A., Sixma, T. K., Wilson, K.S., et Lamzin, V. S. (1997) *Acta Cryst.* **D53**: 448–455;

– Primo-Parmo, S.L., Sorenson, R.C., Teiber, J. et La Du, B.N. (1996) *Genomics* **33**(3), 498-507;

– Ramsdell R. (1999) *Anna J.* **26**(3): 346-7;

– Reddy, S.T., Wadleigh, D.J., Grijalva, V., Ng, C., Hama, S., Gangopadhyay, A., Khorsan, R., Shih, D., Lusis, A.J., Navab, M. et Fogelman, A.M. (2001) *Arterioscler Thromb Vasc Biol* **21**(4): 542-7;

– Rodrigo, L., Gil, F., Hernandez, A.F., Marina, A., Vazquez, J. and Pla, A. (1997) *Biochem. J.* **321**, 595-601;

– Smolen A, Eckerson HW, Gan KN, Hailat N, La Du BN. (1991) *Drug Metab Dispos.*, **19**: 107-112;

– Sorenson, R.C., Primo-Parmo, S.L., Kuo, C.L., Adkins, S., Lockridge, O. and La Du, B.N. (1995) *Proc. Natl. Acad. Sci.* U.S.A. **92**(16), 7187-7191;

– Weeks, C.M. & Miller, R. (1999) *J. Appl. Cryst.* **32**, 120-124.